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<b>14. ABSTRACT</b> During the present funding cycle, we have used in vivo and in vitro approaches to determine whether the endogenous cannabinoid system can be targeted to treat breast cancer. Considerable effort was focused on developing a preclinical model to induce mammary tumors in mice. Oral treatment of the carcinogen, DMBA, led to steady rate of tumors appearance in the mammary region of all female control C57Bl/6 mice within approximately 40 weeks. In contrast, tumor development was substantially delayed in female mice lacking fatty acid amide hydrolase (FAAH), the primary enzyme responsible for catabolism of the endogenous cannabinoid anandamide. We have excised these tumor cells and have been growing them in culture. Once the cells are verified as adenocarcinoma (breast) through H&E staining, we will evaluate them in in vitro assays of tumor proliferation and invasion, as well as implant them in C57BL/6 mice and genetically modified mice to increase throughput of the in vivo experiments. In other studies, we have begun examining the effects of cannabinoids on the proliferation and invasion of the following human breast cell lines: MCF-7, MCF-10a, and MDA-MB-231. Through these complementary in vivo and in vitro approaches, we will determine whether increasing endocannabinoid signaling has preventative, antiproliferative, or anti-invasion effects on breast cancer, as well as determine the mechanism of action.					
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## Introduction

$\Delta^9$ -tetrahydrocannabinol (THC), the chief psychoactive constituent of marijuana, as well as other naturally occurring and synthetically derived cannabinoids possess potential therapeutic effects related to cancer treatment, including reduction in nausea and vomiting associated with cancer chemotherapy (Kluin-Neleman et al., 1979), pain relief in cancer patients (Noyes et al., 1975), and antineoplastic activity in mice (Munson et al., 1975; White et al., 1976). These compounds are known to bind to CB<sub>1</sub> (Matsuda et al., 1990) and CB<sub>2</sub> (Munro et al., 1993) receptors. However, there is little enthusiasm for the development of mixed CB<sub>1</sub>/CB<sub>2</sub> receptor agonists for therapeutic uses because of their marijuana-like and psychomimetic effects. An alternative promising approach is the indirect stimulation of cannabinoid receptors by elevating the endogenous cannabinoids *N*-arachidonoyl ethanolamine (i.e., anandamide; (Devane et al., 1992)) and 2 arachidonyl glycerol (2-AG; (Mechoulam et al., 1995; Sugiura et al., 1995)) by inhibiting their respective catabolic enzymes fatty acid amide hydrolase (FAAH; (Cravatt et al., 1996)) and monoacylglycerol lipase (MAGL; (Goparaju et al., 1999)). Accordingly, the purpose of this project is to determine whether elevating endogenous anandamide through blockade of FAAH will prevent tumor genesis as well as produce antiproliferative and decrease tumor invasiveness. In the first series of studies, we are using 7,12-dimethylbenz[a]anthracene (DMBA), an established animal model of induced breast cancer, to determine whether elevation of anandamide reduces tumor genesis and proliferation. In the second series of experiments, we are examining whether FAAH inhibitors and cannabinoid receptor agonists will reduce proliferation and invasion of human breast cancer lines. Collectively, these work will establish whether the endogenous cannabinoid system is a viable target to treat breast cancer.

## Body

### WHOLE ANIMAL STUDIES: DMBA-INDUCED MAMMARY TUMORS

#### Methods

**Subjects** - Subjects consisted of female C57BL/6J (Jackson Laboratory, Bar Harbor, ME) as well as the following transgenic lines: FAAH (-/-), FAAH-NS (i.e., FAAH is exclusively expressed in the nervous system), CB<sub>1</sub> (-/-), and CB<sub>2</sub> (-/-) mice, and age-matched wild type mice from the Center Transgenic Colony at Virginia Commonwealth University. FAAH-NS have wild type levels of AEA in the neural tissues but highly elevated levels in peripheral tissue (Cravatt et al., 2004). Each of the transgenic lines were backcrossed onto a C57BL/6J (13 generations), except for the CB<sub>2</sub> (-/-) mice, which were backcrossed for 5-6 generations. Mice were housed in a temperature (20-22 °C) and humidity controlled, AAALAC-approved facility, with *ad libitum* access to food and water. Subjects weighed approximately 18-20 g, were housed 4-6 mice per cage, and maintained on a 12:12 light cycle. All experiments were approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University.

**Tumor Induction**— The use of 7,12-Dimethylbenz(a)anthracene (DMBA) is commonly administered via gavage and leads to tumor development within approximately 90 days in rats. However, C57BL/6J mice are known to be resistant to tumor development. Given the anticipated long period of time for DMBA-induced tumors to develop (Aldaz et al., 1996), two approaches were used to induce tumors with this carcinogen. The first set of experiments used the subcutaneous (s.c.) route of administration at the suggestion of a collaborator R. Mehta and the second set of experiments administered DMBA via gavage (Aldaz et al., 1996).

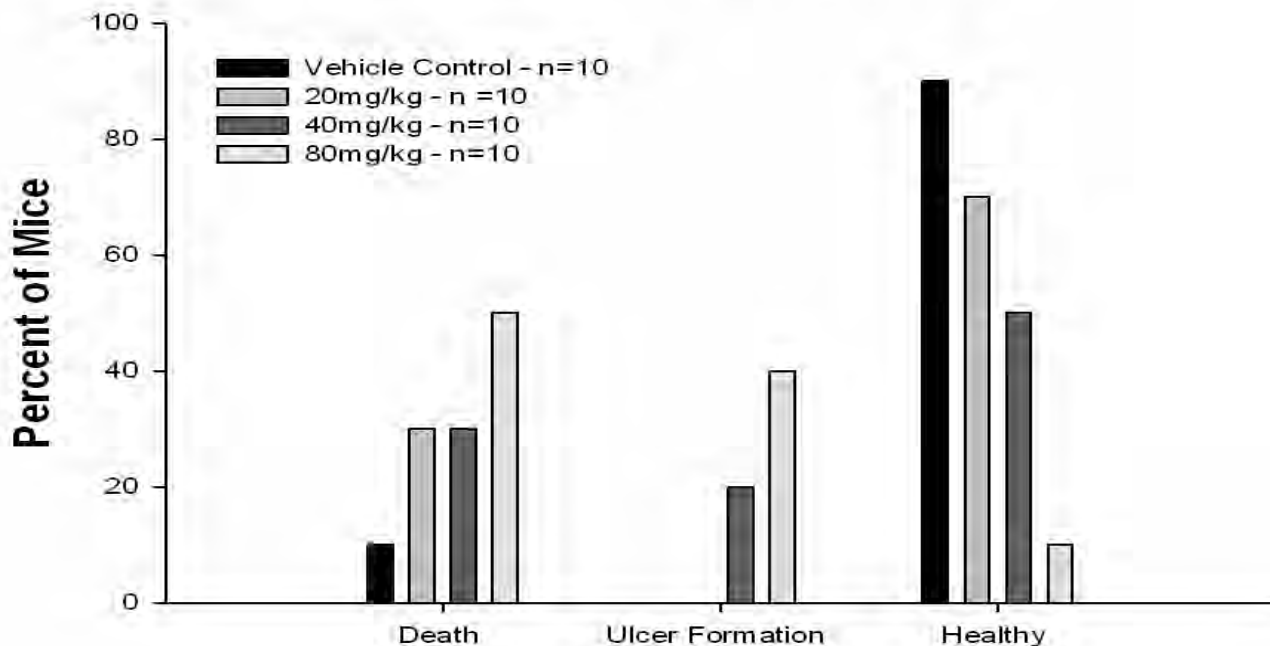
**Tissue Removal and Preservation** – *Animals were euthanized using CO<sub>2</sub> asphyxiation. The tissues were surgically excised and immediately placed in a 10% formalin solution at a solution volume that was at least ten times the volume of the tissue sample. Tissue samples were also sectioned when necessary so that they were approximately 0.5 cm wide to allow rapid formalin perfusion.*

**Experiment 1. Mammary Cancer induction via DMBA s.c. administration:** The needle was inserted in the lower abdomen near the anus and moved parallel to the abdomen until the tip was centered under the lower right nipple. Approximately 0.1 ml of vehicle or DMBA was injected and the needle was removed slowly to prevent leakage from the site. In the first experiment female C57BL/6J mice received cottonseed oil vehicle or DMBA (20, 40, or 80 mg/kg). In the second evaluated 20 mg/kg DMBA in FAAH (-/-) and (+/+) mice, CB<sub>1</sub> (-/-) and (+/+) mice, and CB<sub>2</sub> (-/-) and (+/+) mice.

## Experiment 1 Results

**Experiment 1.1 Induction of mammary tumors using s.c. administered DMBA:** This experiment evaluated the dose-response relationship of DMBA given via the s.c. route of administration. The experiment was concluded at 13 weeks because of considerable toxicity observed in the DMBA-treated mice. No palpable tumor growth was detected. In addition, DMBA led to dose-dependent development of ulcers at the site of injection and lethality (see Figure 1). Likewise, the overall health of the mice, which was based on their lack of ulcers, normal cage behavior, and quality of fur, was adversely affected by DMBA in a dose-dependent manner.

### DMBA Dose Response

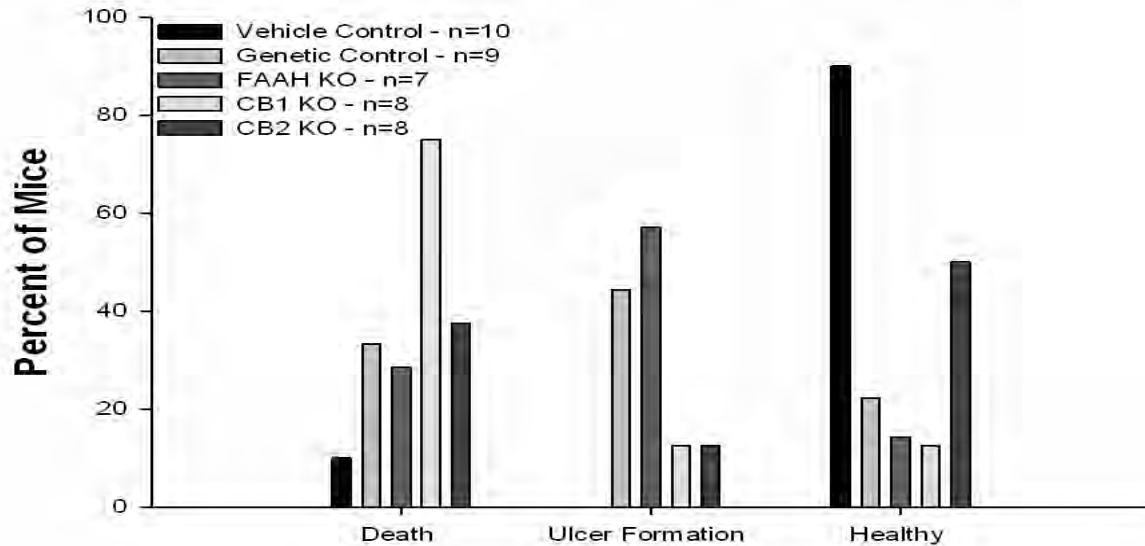


**Figure 1.** Overall evaluation of the effects of s.c. administration of DMBA (20, 40, or 80 mg/kg) in C57BL/6J mice. The experiment was concluded at 13 weeks post-DMBA administration.

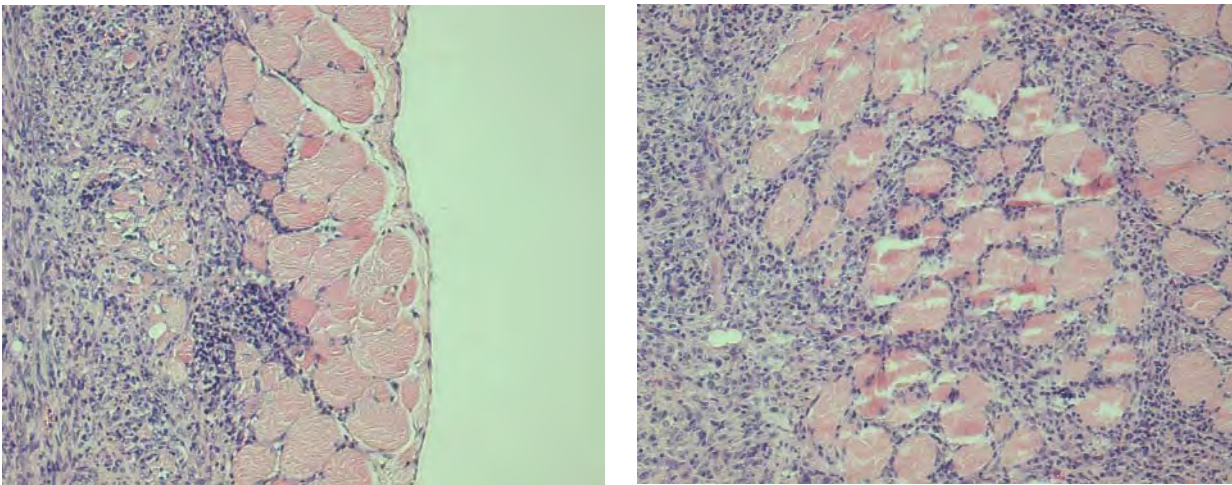
**Experiment 1.2: Effects of s.c. DMBA in FAAH (-/-), CB<sub>1</sub> (-/-), and CB<sub>2</sub> (-/-) mice.** Based on the dose-response DMBA experiment, we selected 20 mg/kg DMBA to assess whether tumor development would be altered in FAAH (-/-), CB<sub>1</sub> (-/-), and CB<sub>2</sub> (-/-) mice as well as their wild type control mice. This dose of DMBA was selected in an effort to minimize the overall toxicity. Again, no palpable tumors were observed following a 12 week observation period, though DMBA increased mortality rates and ulcer formation. The incidence of morbidity and mortality was highest in CB<sub>1</sub> (-/-) mice (see Figure 2).

At the conclusion of the experiment, tissue was excised from the mammary pad around the sight of injection for histology. Using H&E staining, which differentially stains the nucleus (purple) and cytoplasm (pink), we identified four samples that potentially could be malignancies. As can be seen by the representative samples depicted in Figure 3, darker purple stained cells appear to have infiltrated the lighter pink stained tissues, which is a hallmark feature of malignant tumors. Unfortunately, since no palpable tumor masses were detected, we cannot definitively conclude whether DMBA led to carcinoma, though it is possible that these abnormalities were detected before they actually formed into palpable tumors.

## Genotype Effects to DMBA



**Figure 2.** Percentage of FAAH (-/-), CB<sub>1</sub> (-/-), CB<sub>2</sub> (-/-), and wild type control mice that died, developed ulcers at the site of injection, and were healthy in appearance following s.c. administration of DMBA (20 mg/kg). The experiment was concluded at 13 weeks post-DMBA administration.



**Figure 3.** Representative sections of tissue excised from the mammary pads of C57 mice treated with s.c. DMBA (80 mg/kg) and euthanized at 13 weeks following H&E staining. Note that dark purple stained cells (nucleus) appear to have infiltrated the lighter pink stained tissues (cytoplasm), which is a hallmark feature of malignant tumors. Magnification was 20X.

### Experiment 2 Induction of mammary tumors using *gavage administered DMBA*

**Rationale for methodology changes:** After the initial experiments described above, it was clear that the occurrence of possible malignancies (see figure 3) suggested that DMBA was still potentially a viable model to induce mammary tumors in mice, but the high incidences of mortality and morbidity required changes to the methods. Administration of DMBA by gavage is more common than s.c. administration, but tumor development is a very long process in mice (Aldaz et al., 1996). Thus, we opted to employ the gavage route of administration to limit the incidence of toxicity and death. In addition, we administered progesterone along with the DMBA because it has been reported to increase the rate of mammary tumor development, while reducing toxic effects of DMBA (Aldaz et al., 1996).

**Tumor Induction** - Prior to surgery the back of the neck of the mouse was shaved and then washed with Betadine solution and ethanol. The mice were anesthetized using isoflurane and then using sterile surgical equipment a small one inch incision was made just below the ears to prevent excessive scratching during healing. A 25 mg 60 day release progesterone pellet was inserted subcutaneously into the incision and then moved down the back. The incision was then closed using staples. The isoflurane was removed and the animals were returned to their home cages. Drinking water mixed with acetaminophen (2.5 mg/ml of water) was made available ad libitum for one week to reduce any post-surgical discomfort. The mice were given 21 days to recover from the progesterone pellet implantation before beginning DMBA dosing.

Gavage was performed using a syringe with a rounded tip to prevent damage to the esophagus. Each mouse was given 50 mg/kg DMBA dissolved in cottonseed oil on days 1, 8, 22, and 29 in an injection volume of 0.1 ml. The following two control groups were included: 1) a vehicle control group that was given cottonseed oil with no progesterone pellet implanted; and 2) a progesterone control group that was implanted with a progesterone pellet, but received cottonseed oil instead of DMBA. Following the last dose each mouse was palpated weekly. Tumor formation was expected to occur by 24 weeks (Aldaz et al., 1996).

Finally, in an effort to increase the rate of tumor formation, but to reduce toxicity, we examined the effects of s.c. DMBA (5, 10 and 20 mg/kg) on mammary tumor development in progesterone pelleted C57BL/6J mice. It was our hope that the progesterone would allow us to

## Results

The purpose of this experiment was two-fold. First, we wanted to optimize the DMBA to increase the incidence and rate of tumor development, while limiting DMBA-induced toxicity. Second, we wanted to examine whether mice with increased levels of anandamide (i.e., FAAH (-/-) mice) or disrupted cannabinoid receptor signaling (i.e., CB<sub>1</sub> (-/-) and CB<sub>2</sub> (-/-) mice) would display altered rates of tumor development. Regarding our first aim, we did make a change to the model mid experiment. In these experiments, we extended the duration of the study from 24 weeks to 45 weeks, which resulted in the majority of mice developing tumors. In addition, we are developing cell lines from the tumors, which will be discussed later.

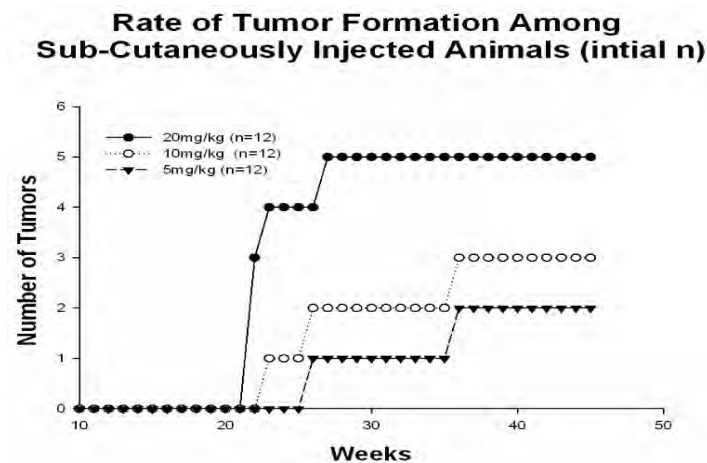
Each of the treatment groups of mice in Experiment 2 are listed in Table 1. Also listed are the values: 1) number of mice in each group; 2) number of deaths related to improper gavage technique combined with DMBA toxicity; 3) number of other deaths (i.e., DMBA-related toxicity and undetermined); 4) number of mice that survived to week 45; and 5) number of mice that developed tumors. Unfortunately, in this study a large number of mice died because of improper gavage technique. These mice died within several days of the first gavage injection. The CB<sub>2</sub> (-/-) group was particularly decimated by this problem. Thus, no meaningful results can be concluded about the impact of deleting the CB<sub>2</sub> receptor on DMBA-induced tumors. Once the problem was detected, the gavage technique was mastered and no additional gavage-related deaths occurred.

**Table 1.** Experiment 2 Results: The number of mice in each group that died because of improper gavage technique or other causes, and the number of surviving mice that developed tumors by week 45.

Injection Route	DMBA dose (mg/kg)	Proges.	Mouse Type	Initial n	# Gavage-related deaths	# Other deaths	# survived at 45 weeks	# surviving mice with tumors
s.c.	20	Yes	C57	12	0	4	8	5
s.c.	10	Yes	C57	12	0	6	6	3
s.c.	5	Yes	C57	12	0	3	9	2
gavage	0	No	C57	12	1	1	11	0
gavage	0	Yes	C57	12	2	1	9	2
gavage	0	Yes	CB <sub>1</sub> (+/+)	5	2	1	2	1
gavage	50	Yes	CB <sub>1</sub> (+/+)	5	3	0	2	2
gavage	50	Yes	CB <sub>1</sub> (-/-)	12	5	5	2	2
gavage	50	Yes	CB <sub>2</sub> (+/+)	4	2	1	1	0
gavage	50	Yes	CB <sub>2</sub> (-/-)	11	9	1	1	1
gavage	50	Yes	FAAH (+/-)*	10	2	2	6	6
gavage	50	Yes	FAAH-NS	10	1	1	8	7
gavage	50	Yes	FAAH (-/-)	10	3	1	6	6
Total				127	30	27	71	37

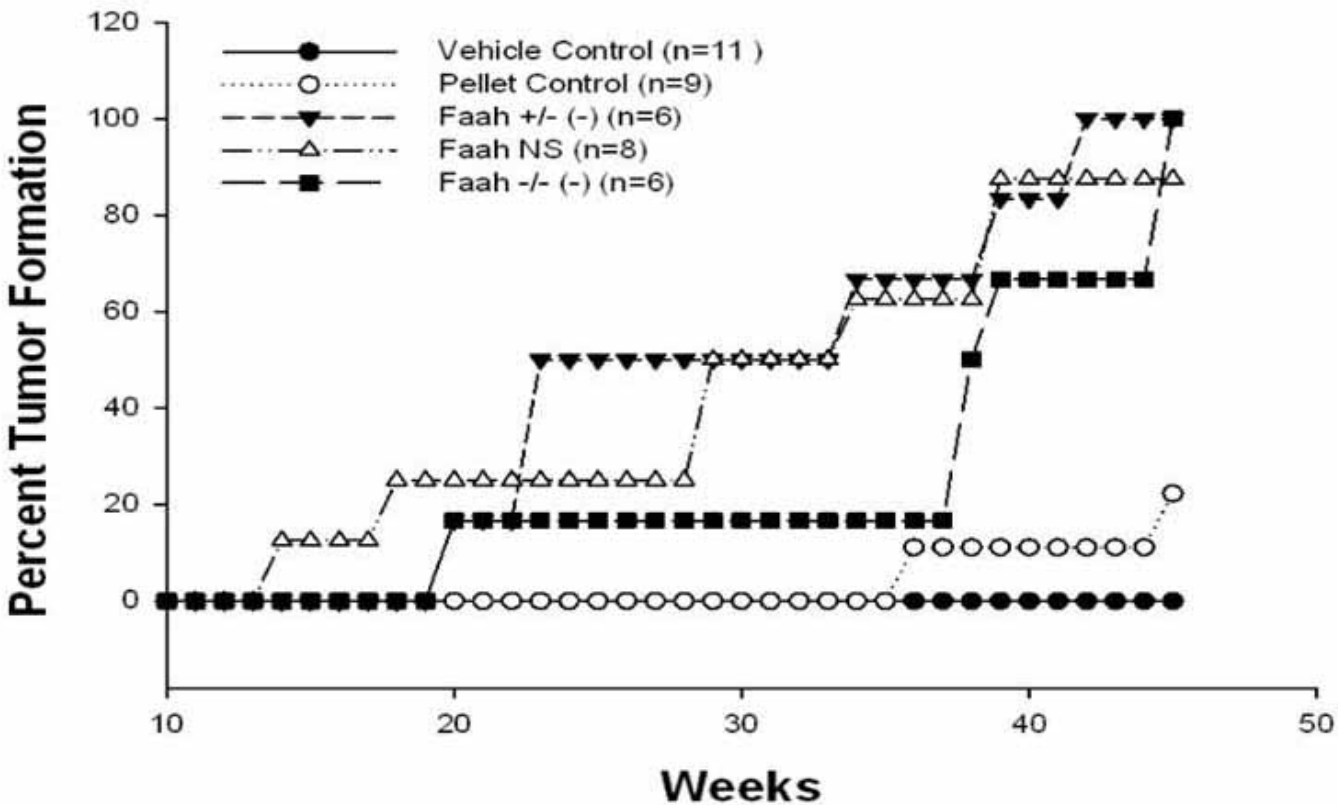
\* FAAH (+/-) mice were used as controls because of the husbandry used to derive FAAH-NS and FAAH (-/-) mice from the same litter. It is important to note, that although FAAH (+/-) mice express half the amount of this enzyme as wild type mice, and have wild type levels of anandamide and normal CB<sub>1</sub> receptor function.

Subcutaneous DMBA dose-dependently increased the percentage of mice developing tumor and the rate of tumor development (Figure 4), but these effect occurred in conjunction with toxicity and mortality. Therefore, future studies will use gavage administered DMBA in progesterone pelleted mice.



**Figure 4.** Subcutaneously administered DMBA dose-dependently increased the rate of tumor development. However, this effect was also associated with an increase in toxicity and death (see Table 1).

underlying mechanism for this apparent phenotypic delay in tumors is not addressed in the present study, it could potentially involve the hypothalamic-pituitary-adrenal (HPA) axis (Hill et al., 2009).



**Figure 5.** Rate of Tumor Formation Among FAAH Transgenic mice. FAAH (-/-) mice display a delayed rate of tumor development induced by DMBA compared to FAAH (+/-) control and FAAH-NS (i.e., FAAH is exclusively expressed in nervous system). DMBA was administered via gavage in mice implanted s.c. with progesterone.



Because of the small sample size in the CB<sub>1</sub> (-/-) and CB<sub>2</sub> (-/-) mice (Table 1), we are unable to make any conclusions on the impact of cannabinoid receptor deletion DMBA-induced mammary tumors. We are currently increasing the number of female CB<sub>1</sub> (-/-) and CB<sub>2</sub> (-/-) mice in our colony to test whether the cannabinoid receptor plays a protective role.

A critical question regarding the face validity of the DMBA-induced breast cancer model is whether the tumors are mammary or of other derivation. Female mice do not have breast tissue, but instead have a series of thin mammary pads that cover the entire underside of their body. Accordingly, we have differentiated these the tumors based on anatomical location (e.g., nipple area vs. back). However, tumors on the underside could be derived from skin as well as mammary. Thus, histology is critical to determine whether the tumors are mammary. Using basic H&E stain as well as cell morphology, tissue morphology, tissue type we are in the process of determining whether the excised tissue is cancerous and its type (e.g., squamous carcinoma (skin) or adenocarcinoma (mammary)). In addition, we are using a battery of biological markers, including p63, a nuclear protein commonly expressed by mammary duct epithelial cells, but lost when developed into cancer. In addition we are examining cytokeratins 5, 6, and 7, which are associated with mammary tissue and can be either lost or present in breast cancer based on the type of cytokeratin. Our last stains, mucocarmine and PADS, are aimed at detecting the presence of mucus which is secreted by all glandular tissue like the mammary glands. If a tumor is producing mucus it is a strong indicator that it was originally derived from mammary tissue. The basic histological profile indicating mammary tumors would show mammary ducts visible in the H&E stains, p63 and cytokeratin 5/6 negative, cytokeratin 7 positive, and at least one of the mucus stains positive. However it should be mentioned DMBA may induce multiple forms of cancer, including being an adenocarcinoma with squamous carcinoma characteristics. Thus, the array of markers are being employed to prevent false positives. During the present funding period, we have conducted H&E stains on all samples to confirm the presence of cancerous tissue. However, we are currently assessing the remaining biological markers and will determine if they are mammary or skin cancer within the next few weeks.

### **Section 1.3 – Cell lines derived from tumors extracted from mice**

#### **Reasoning for development of cell lines**

4T1 cells are mammary tumor cell line derived from the Balb/c strain of mice. When these cells are re-introduced into the Balb/c mice they are not rejected by the immune system, allowing the tumor cells to proliferate in a manner that is similar to that of metastatic disease in patients (McKallip et al, 2005)

This approach is of benefit because xenograft models using human tumor cell lines grown in mice require suppression of the mouse immune systems. The obvious limitation of this model is that it eliminates potential interactions between pharmacological treatments and the immune system. However, we do recognize that a mouse breast tumor is not necessary a direct reflection of breast cancer in humans.

Unfortunately, the genetically manipulated animals that are at our disposal are not from the Balb/c strain, and therefore we are unable to use the 4T1 breast tumor model. We also have not been able to identify any other mammary tumor cell lines that can be transplanted into the C57 mice, which is the strain of our genetically manipulated animals. We therefore decided that it would prove useful to develop a mammary tumor cell line as an additional tool for alternative future studies as well as a backup approach in the event that the DMBA model in mice proved to be unfeasible.

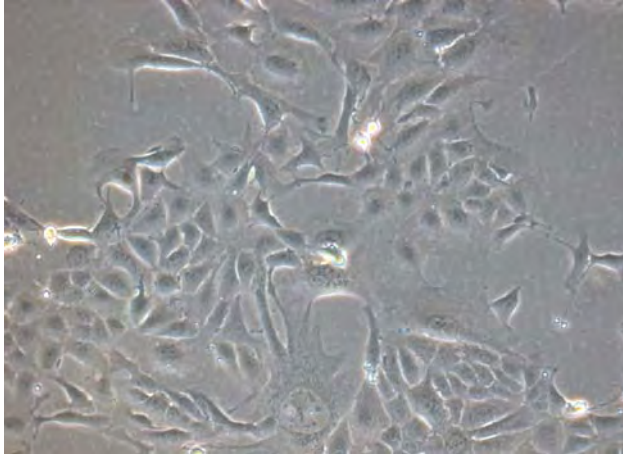
#### **Methods**

**Cell line development** - Animals are sacrificed using CO<sub>2</sub> asphyxiation and the tumors are excised under sterile conditions and placed in a cell culture dish, where they are divided into two parts. One part is provided for histological analysis. The remaining tissue is minced, and covered with our standard growth media (RPMI with 5% fetal bovine serum, 5% bovine calf serum, and 1% pen/strep). The media contains 25μg fungizone to ward off fungal infection and approximately 5mg of collagenase to digest the tissue. The cells are washed with PBS and the media is replaced every 3 to 4 days. The collagenase is maintained in the media for the first two washes while the fungizone is maintained throughout the process. Once a significant number of cells can be seen proliferating from the tumors, they are transferred to a new culture dish after trypsinization and cultured normally depending on their individual growth rate. Fungizone is maintained in the media into the third passage to ensure that dormant infections are not transferred.

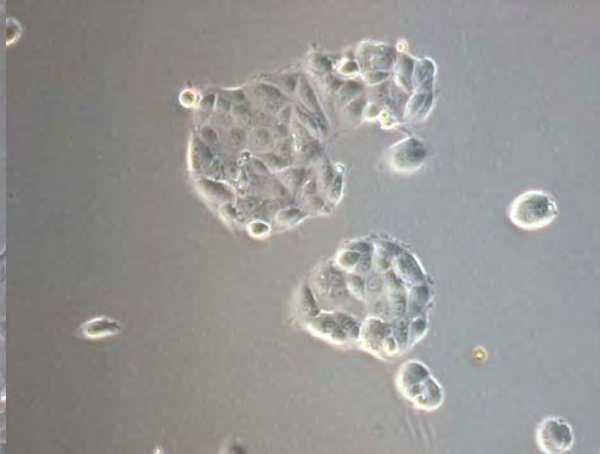
#### **Future plans**

We currently have removed four tumors that we were able to develop into proliferating cell lines. These are shown below (Figure 6):

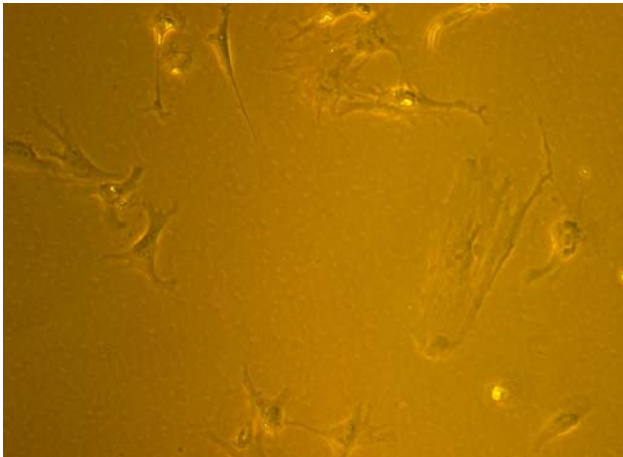
BR4 – P – C57



BR15 – P – C57



BR16 – P – C57



BR18 – P – C57

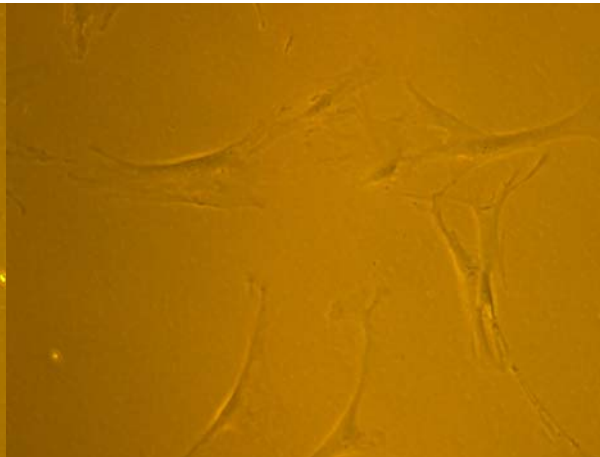


Figure 6. Images of cells derived from carcinogen induced tumors in mice as described in text.

Of these four cell lines we have cultured and frozen samples for the BR4 and BR15 strain and we are in the process of doing the same for the remaining two cell lines. Following the final establishment of the cell lines, we plan to confirm that they are in fact mammary tumors. If these are confirmed, we will begin characterization of the cells growth and characteristics both in vivo and in vitro.

## Section 2.1 – First attempt at in vitro cell transformation

### Methods

**Cell growth** – All cell lines (Mcf-7, Mcf-10a, and MDA-MB-231) are grown in a 37<sup>0</sup> incubator and passed twice weekly. The Mcf-7 and MDA cells are maintained in our standard growth media (RPMI with 5% fetal bovine serum, 5% bovine calf serum, and 1% pen/strep). Our Mcf-10a cells are grown in specialized media: DMEM/F12, 5% horse serum, 1% pen/strep, 10ug/ml insulin, 100ng/ml cholera toxin, 20ng/ml EGF, and 500ng/ml hydrocortisone.

**Transformation** – The cells are seeded into six well plates at low density (approximately 10,000 cells per well) and allowed to grow for one week before passage. During that week, drug treatment is administered twice for 24hrs each. Treatment one is administered at 72 hours and removed at 96 hours, and treatment two is administered at 120 hours and removed at 144 hours. At the end of week one, the cells are passed back into six well plates for a second week of treatment, identical to the first. At the end of the second week of treatment, the cells are passed into normal culture flasks and allowed to grow to sufficient numbers for analysis, which

includes measurement of anchorage independent growth, invasiveness, and growth rates under various conditions. Analysis begins by the end of week three after initiation of transformation.

**Anchorage independent growth (AIG)** – The bottoms of six well plates are coated with 1.5ml of 12mg/ml poly-hema dissolved in 95% ethanol. The ethanol in the plates is allowed to evaporate overnight, while under UV lights for sterilization, leaving a clear coating on the bottom of the plate that prevents cell attachment. On day one of the experiment, 100,000 cells are placed in each well with their normal growth media and allowed to grow in a 37<sup>0</sup> incubator. At days 4, 8, 12, 16 and 22, the cells are counted using a hemocytometer to establish any changes in the number of cells within the well. More than three time points has been found to be necessary because due to the duration of the experiment and frequent fungal contamination. At each time point, the cells from each condition of the transformation are seeded in triplicate and the average of the three wells is taken. MDA-MB-231 cells are used as a positive control in this experiment.

**Boyden chamber invasion assay** – The Boyden chamber inserts are placed into 24 well plates, allowed to soak in media for 5 minutes, and then coated with matrigel diluted 1:6 using media. The matrigel is allowed to set for at least 20 minutes but must be used within 2 hours to start the assay. Trypsinized cell samples are spun down and resuspended at 1x10<sup>6</sup>cells/ml in serum free media for all conditions. 200uL of the cell suspension is then placed on top of the matrigel covered insert suspended in the 24 well plate. Serum free media is also placed in the well under the matrigel insert. The cells are then allowed to sit for 24 hours in a 37<sup>0</sup> incubator before analysis. After 24 hours, the invasive cells should have moved through the matrigel and from the top to the bottom of the boyden chamber insert. At this point, the insert is washed with PBS and the cells are fixed with glutaraldehyde. The fixed cells are then stained with toluidine blue and can be counted under a microscope. For quantification, multiple pictures are taken of the stained cells in various fields of the insert to determine the level of invasion. In this assay MDA-MB-231 cells can be used as a positive control and Mcf-7 cells can be used as a negative control.

**Growth assay** – On day one of the experiment, 10,000 cells are placed in each well of a six well plate with their normal growth media and allowed to grow in a 37<sup>0</sup> incubator. Three wells are set up for each condition at each time point. On day two, the cells are counted using a hemocytometer to obtain a baseline cell number. Relevant time points will be either days 2, 3, 4, and 5 or days 2, 5, and 8.

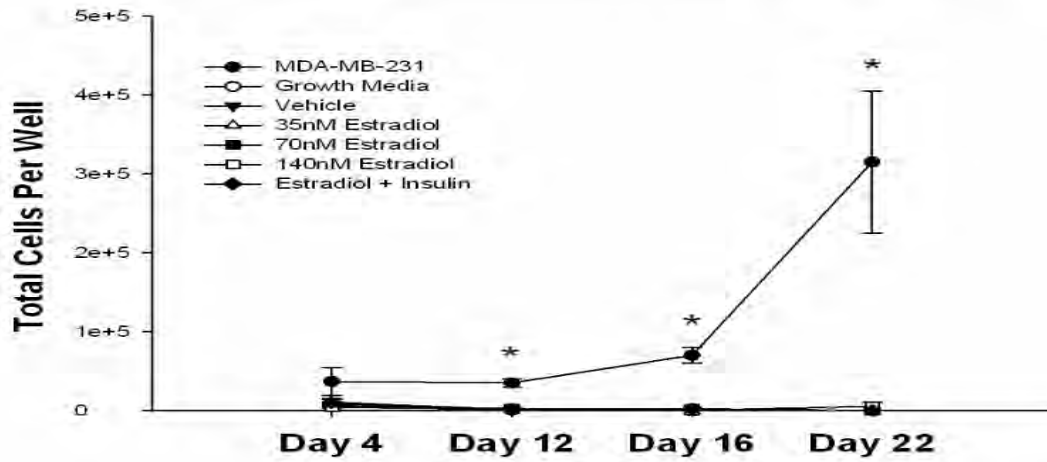
**EXPERIMENTAL DESIGN.** Mcf-10a cells are a spontaneously immortalized breast epithelial cell line that can theoretically be transformed to a tumorigenic state when exposed to carcinogens (Russo et al, 2006). Following the basic transformation protocol described above, Mcf-10a cells were exposed to: growth media alone, media containing vehicle, 35nM Estradiol, 70nM Estradiol, 140nM Estradiol, or 70nM Estradiol plus 100nM Insulin.

## Results

We first analyzed the cells' capacity for anchorage independent growth (AIG) (Figure 7). The normal Mcf-10a cells are not capable of growing in an environment where they cannot adhere to a surface. Therefore cells that gain the capacity for growth in this environment would be indicative of transformation. However, the cells showed no signs of growth while the MDA-MB-231 cells (a positive control) grew exponentially over the course of the experiment. These observations validate the AIG assay, but indicate that the Mcf-10a cells have not undergone transformation.

We further analyzed the invasiveness of cells after exposure to the putative carcinogenic stimuli using the Boyden chamber invasion assay. MDA-MB-231 cells were again utilized as a positive control and Mcf-7 cells as a negative control as Mcf-7 cells are not an invasive cell line. Mcf-10a cells have a low level of invasiveness but it has been show that estradiol treatment can enhance their ability to invade in the boyden chamber invasion assay (Russo et al, 2006). Representative images of the Boyden chamber are shown in Figure 8 for all the conditions as well as the positive and negative control. When quantified, it was evident that there was no significant enhancement of the invasiveness of the Mcf-10a cells (Figure 9).

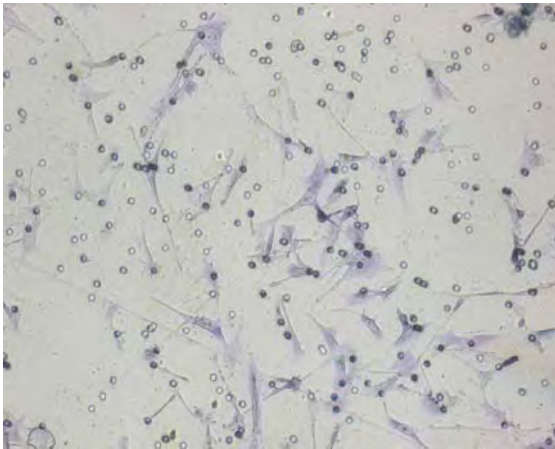
## Anchorage Independent Growth Rates



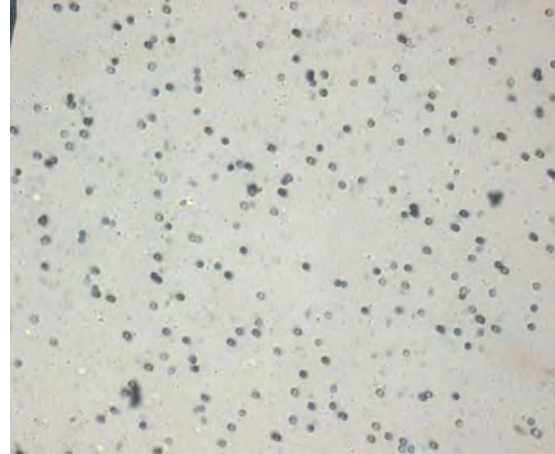
### ANCHORAGE INDEPENDENT GROWTH RATES

Figure 7. Anchorage independent growth for the transformed Mcf-10a cells. Each time point is expressed as the average number of cells counted between three wells. Here MDA-MB-231 cells were used as the positive control against all conditions of transformation. \*=significantly different than Growth Media group (ANOVA,  $p < 0.05$ )

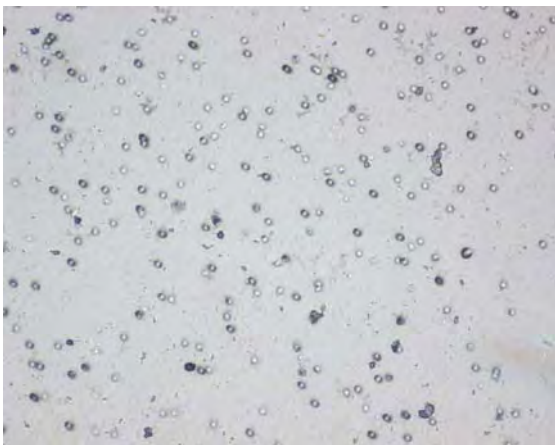
A) MDA-MB-231



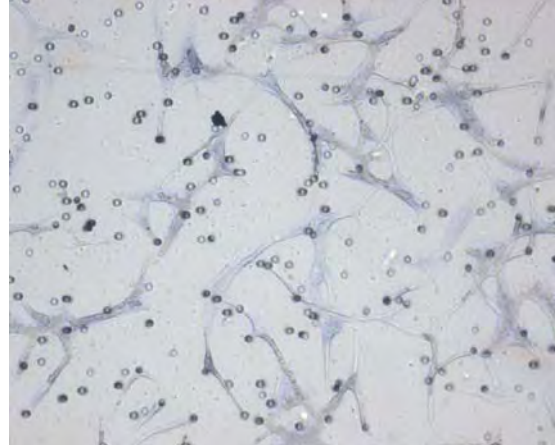
B) Mcf-7



C) Mcf-10a – growth media



D) Mcf-10a – vehicle control



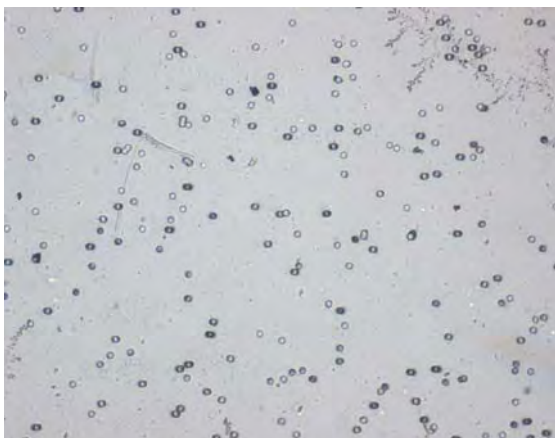
E) Mcf-10a – 35nM estradiol



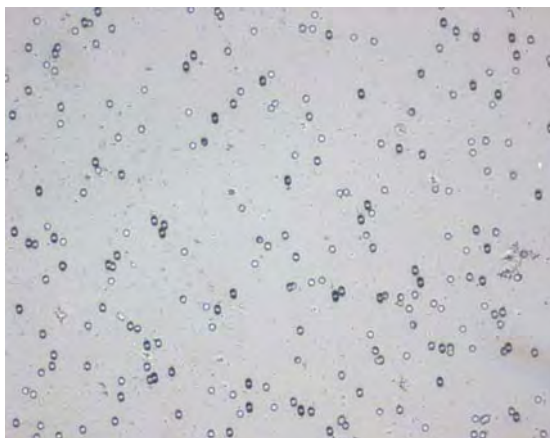
F) Mcf-10a – 70nM estradiol







G) MCF-10A – 140nM estradiol



H) MCF-10A – estradiol and insulin

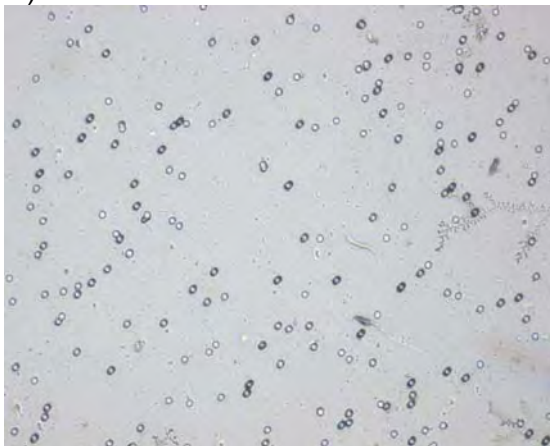
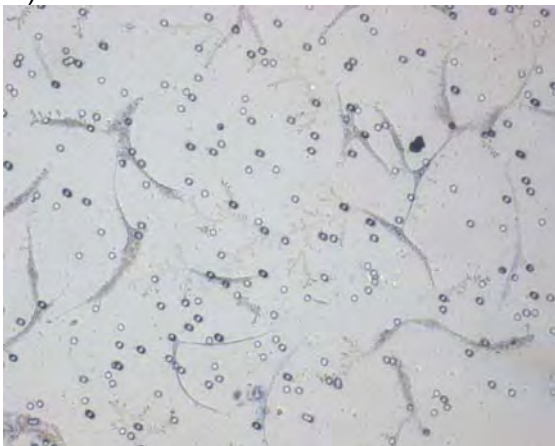


Figure 8. Representative images of breast tumor cells invasion assessed using the Boyden Chamber assay. Experimental details are provided in the text.

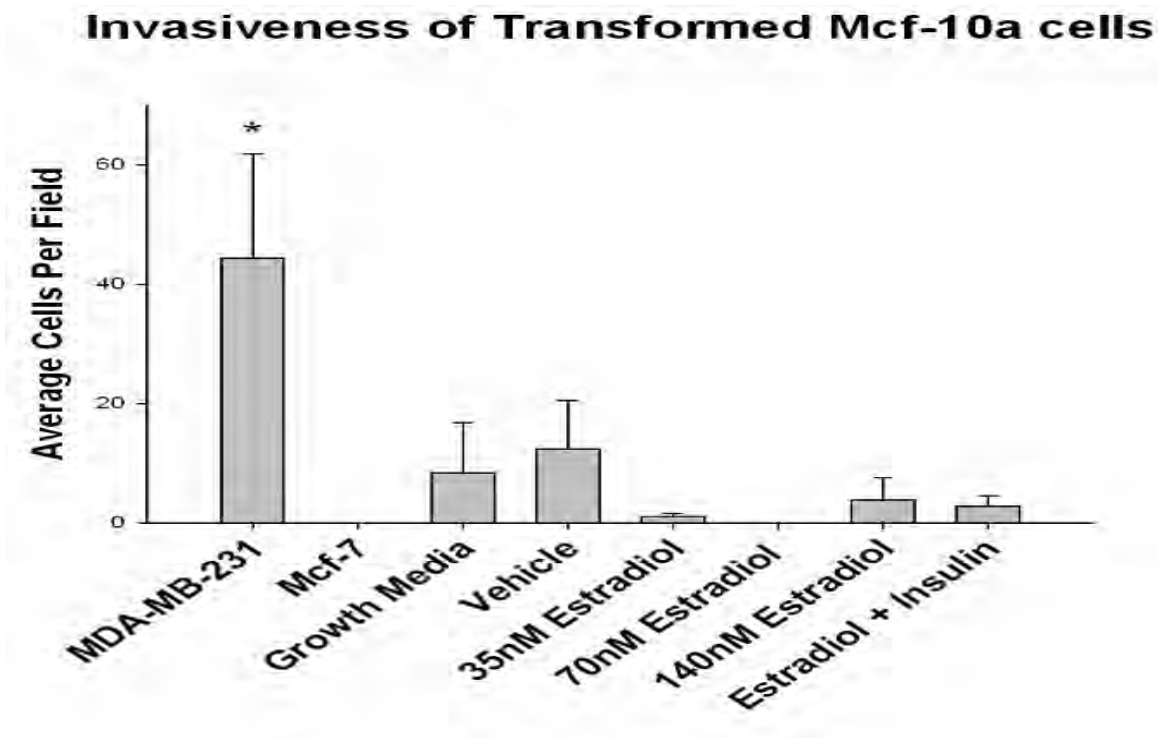


Figure 9. Invasive capacity for the transformed Mcf-10a cells. Each data point is expressed as the average number of cells counted between 5 pictures of the Boyden chamber. Here the MDA-MB-231 cells are used as our positive control and the Mcf-7 cells are used as a negative control. \*=significantly different than Growth Media group (ANOVA,  $p < 0.05$ )

Finally, we examined the growth rate of the Mcf-10a cells under various conditions (Figure 10 A, B and C). Specifically, we assessed proliferation in normal growth media (10A) as well as proliferation that was independent of exogenous growth factors (insulin, cholera toxin, egf, and hydrocortisone) but in the presence of 5% horse serum (10B). As many breast cancers are found to be estrogen dependent, we hypothesized that transformation with could induce this type of dependence. Consequently, we evaluated the growth rate of the transformed cells after all additional growth factors were removed from the media followed by administration of 70nM estradiol (10C). This last measurement of growth rate was extended to day 8 instead of the previous time point day 5 to ensure there was no delayed effect, but no significant differences were found in any of the measurements of growth rate.

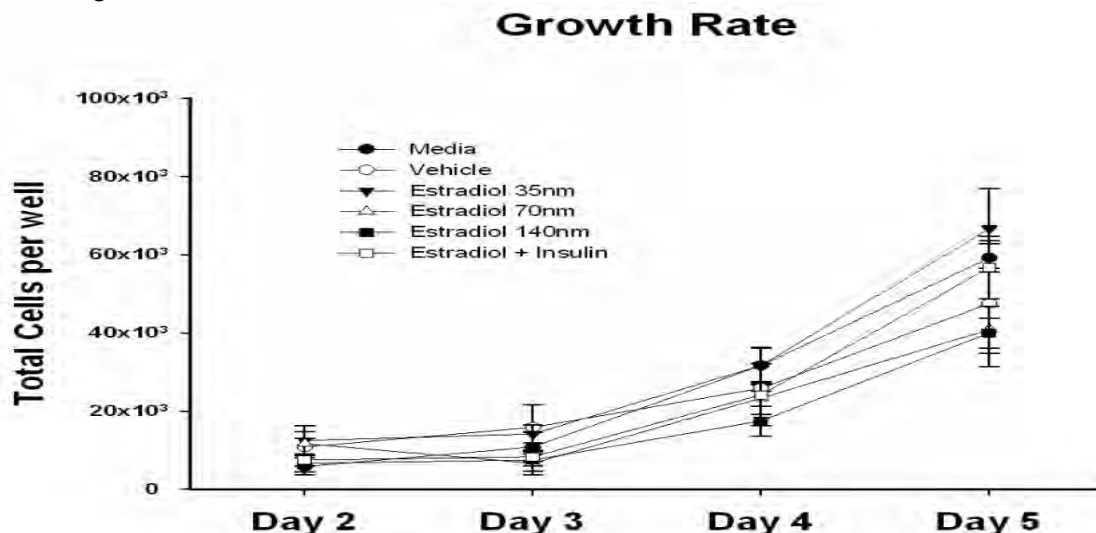


Figure 10A

## Growth Rate minus Growth Factors

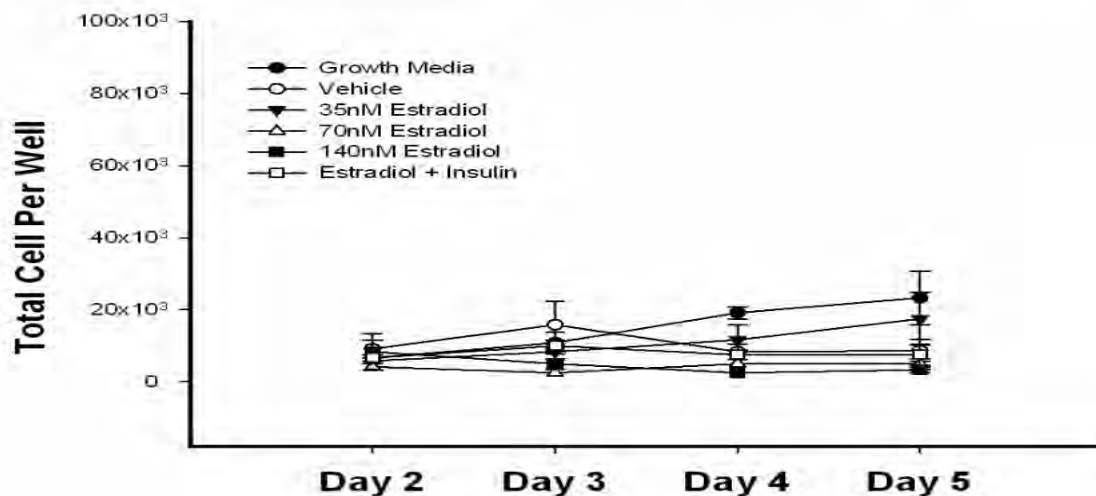
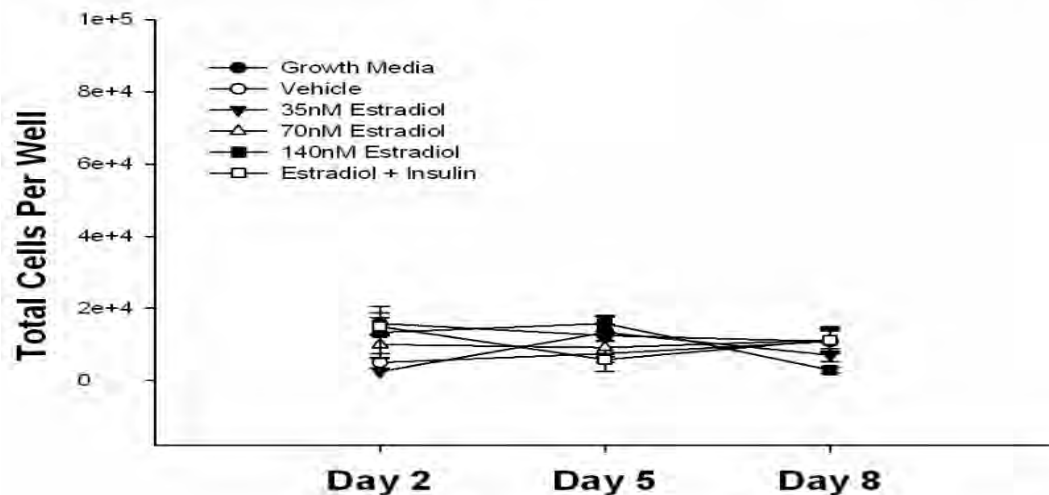


Figure 10B

## Growth Rate with Estradiol in Media



Figure

10C

**Figure 10A.** Growth rate for the transformed Mcf-10a cells. Each time point is expressed as the average number of cells counted between three wells. All cells were allowed to grow in their normal growth media described in the methods. All time points are the mean  $\pm$  se and using ANOVA no significant differences were found.

**Figure 10B.** The capacity of transformed Mcf-10a cells to grow absent their standard growth factors. Each time point is expressed as the average number of cells counted between three wells. All cells were allowed to grow in DMEM/F12 media with horse serum and penicillin/streptomycin described in the methods. All time points are the mean  $\pm$  se and using ANOVA no significant differences were found.

**Figure 10C.** The sensitivity of transformed Mcf-10a cells to the added presence of estradiol in media. Each time point is expressed as the average number of cells counted between three wells. All cells were allowed to grow in DMEM/F12 media with horse serum, penicillin/streptomycin and 70nM Estradiol described in the methods. All time points are the mean  $\pm$  se and using ANOVA no significant differences were found.

## Section 2.2 – Second attempt of in vitro cell transformation

### Reasoning behind method changes

A number of studies have demonstrated that is the smoking related carcinogen, Benz-a-pyrene, (100pM ) could promote transformation (Siriwardhana 2007). Benz-a-pyrene is a polycyclic aromatic hydrocarbon (PAH), which has mitogenic effects and can up regulate the expression of a p450 enzyme that metabolizes these compounds to their reactive epoxide state. Once metabolized, the PAHs are capable of forming DNA adducts that can transform the cells. Mammary epithelial cell transformation has also been reported using the alkylating agent, N-Methyl-N-Nitrosourea (MNU) (Methods in Mammary Gland Biology and Breast Cancer Research )

### Methods

**Transformation** - Estradiol would be administered twice weekly for twenty four hours each administration per week. Treatments would occur at 72 hours and at 120 hours after cells are seeded. This treatment would be repeated for two weeks. Benz-a-pyrene (100pM) would be administered 24 hours after the cells are seeded for the experiment and allowed to remain in the media for 48 hours; it would then be removed from the media for 24 hours, and then for the last 72 hours of the week it would be restored to the media. This week long treatment would then be repeated, resulting in a total of two weeks of treatment with Benz-a-pyrene. MNU, 50ug/ml, would be administered to the cells for 1 hour instead of 24 hours twice weekly over a two week period. Analysis of the cells after transformation will remain the same as in section 2.1, including AIG, Boyden Chamber Invasion, and growth rate measurement.

**Rationale** The enzyme responsible for metabolizing estradiol to its reactive state, where it can then form DNA adducts, is the same one that activates PAHs. We speculated that combining the estradiol and Benz-a-pyrene treatments could enhance transformation. We also added a condition where the cells were exposed to estradiol, Benz-a-pyrene and MNU.

**Results** These studies are currently in progress.

### Key Research Accomplishments

- Almost all C57BL/6J mice developed tumors following modifications to the DMBA-induced model
- Initiated carcinogen-induced tumor growth in mice with genetic modulation of cannabinoid receptors
- Extracted tumors from the mice for development of unique syngeneic breast tumor models
- Observed delay in tumor development in the FAAH (-/-) mice induced by DMBA
- Established assays for breast tumor cell transformation in cell culture
- Evaluated a number of approaches for inducing breast tumor initiation from breast epithelial cells in culture

**Reportable Outcomes: None, at present. Studies are in progress.**

### Conclusions

A DMBA induced model of breast cancer is feasible in C57Bl/6J mice and transgenic micewith genetic modulation of cannabinoid receptors on the C57Bl/6 background.

FAAH (-/-) mice that possess elevated levels of the endocannabinoid anandamide as well as noncannabinoid lipid signaling molecules display a delay in the development of DMBA-induced tumors.

It is likely that we will be able to develop novel syngeneic models of breast cancer that can be used to identify the contribution of cannabinoid signaling to tumor growth and development.

MCF10A breast epithelial cells are relatively refractive to carcinogen induced transformation.



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